RESEARCH ARTICLE

2-D DIGE analysis of the mitochondrial proteome from human skeletal muscle reveals time course-dependent remodelling in response to 14 consecutive days of endurance exercise training

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Received: September 19, 2010
Revised: December 8, 2010
Accepted: January 11, 2011

Adaptation of skeletal muscle to repeated bouts of endurance exercise increases aerobic capacity and improves mitochondrial function. However, the adaptation of human skeletal muscle mitochondrial proteome to short-term endurance exercise training has not been investigated. Eight sedentary males cycled for 60 min at 80% of peak oxygen consumption (VO_{2peak}) each day for 14 consecutive days, resulting in an increase in VO_{2peak} of 17.5 ± 3.8% ($p < 0.01$). Mitochondria-enriched protein fractions from skeletal muscle biopsies taken from m. vastus lateralis at baseline, and on the morning following the 7th and 14th training sessions were subjected to 2-D DIGE analysis with subsequent MS followed by database interrogation to identify the proteins of interest. Thirty-one protein spots were differentially expressed after either 7 or 14 days of training (ANOVA, $p < 0.05$). These proteins included subunits of the electron transport chain, enzymes of the tricarboxylic acid cycle, phosphotransfer enzymes, and regulatory factors in mitochondrial protein synthesis, oxygen transport, and antioxidant capacity. Several proteins demonstrated a time course-dependent induction during training. Our results illustrate the phenomenon of skeletal muscle plasticity with the extensive remodelling of the mitochondrial proteome occurring after just 7 days of exercise training suggestive of enhanced capacity for adenosine triphosphate generation at a cellular level.

Keywords:
2-D DIGE / Biomedicine / Exercise training / Mitochondrial proteome / Skeletal muscle

1 Introduction

Physical inactivity contributes to the development of at least 25 clinical conditions, including some of the most prevalent causes of mortality [1]. A recent meta-analysis reported a lower risk of all-cause mortality and cardiovascular events in individuals with higher cardiorespiratory fitness [2]. Regular physical activity and the preservation of maximal aerobic capacity are consequently considered as critical tools in the amelioration of metabolic dysfunction, prevention of chronic diseases, and promoting longevity [1, 3].

Skeletal muscle shows a remarkable malleability to remodel its phenotype and to adapt functionally in response to contractile stimuli such as regular exercise [4, 5]. This skeletal muscle plasticity is illustrated by the remodelling of muscular force, endurance, and contractile velocity as a

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Abbreviations: ATP, adenosine triphosphate; **CCB,** colloidal Coomassie Blue; **COX,** cytochrome *c* oxidase; **DLD,** dihydrolipoyl dehydrogenase; **ETC,** electron transport chain; **MDH,** malate dehydrogenase; **mi-CK,** mitochondrial creatine kinase; **Mn-SOD,** manganese-superoxide dismutase; **PCr,** phosphocreatine; **PDH,** pyruvate dehydrogenase; **TCA,** tricarboxylic acid; **VO_{2max},** maximum oxygen uptake

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result of alterations in functional demand. These adaptations collectively contribute towards maximizing substrate delivery, respiratory capacity, and contractile parameters during sub-maximal exercise [6]. Maintaining physiological homeostasis and normal cellular function during exercise is dependent on the energetic demands of the muscle being met by a sufficient production of adenosine triphosphate (ATP) by the mitochondria.

The first observation that adaptation to regular endurance exercise increases skeletal muscle oxidative capacity and enhances mitochondrial function was made almost half a century ago [7]. In recent years, mitochondria, owing to their critical role in cellular metabolism and formidable plasticity, have become the centre of attention for research skeletal muscle plasticity in health and disease [8]. Proteomic approaches have identified alterations in mitochondrial protein abundance, indicating the defects in mitochondrial metabolism in skeletal muscle from obese, type 2 diabetic (T2D), and elderly individuals [9–12]. While several metabolic pathways in skeletal muscle have been interrogated in response to exercise training, most of which have traditionally taken a gene-by-gene approach, training-induced remodelling of the human skeletal muscle mitochondrial proteome has not yet been subjected to a proteomic investigation. Therefore, relatively little is known about the global changes in the mitochondrial proteome following exercise training.

In the present study, we describe a proteomic analysis using 2-D DIGE [13] of a mitochondria-enriched protein fraction from human skeletal muscle in response to short-term (14 days) endurance exercise training. Protein labelling with CyDye DIGE fluors is very sensitive, with a detection limit of around 500 pg for an individual protein and a linear response in protein concentration over at least five orders of magnitude [14]. Recently, the first report describing the response of the human skeletal muscle proteome to exercise training emerged [15]. This group detected 256 gel spots proteins of which 20 were proteins exhibiting altered expression after 6 wk of interval training. Relatively few mitochondrial proteins were resolved using this 2-DE approach on whole-muscle lysates, which can attributed to a high abundance of myofibrillar proteins and glycolytic enzymes [15, 16]. Moreover, mitochondrial proteins are difficult to locate on a global proteome due to the sensitivity of the methods to absolute protein abundance [17]. We used the more sensitive 2-D DIGE technique [14] to investigate mitochondrial proteome remodelling in an experimental model known to elicit robust changes in skeletal muscle metabolism and whole-body aerobic capacity [18, 19]. Short-term endurance training resulted in extensive remodelling of the mitochondrial proteome from human skeletal muscle, suggesting increased capacity for ATP provision and re-synthesis, oxygen delivery and antioxidant capacity, and alterations in the molecular machinery regulating mitochondrial protein synthesis, several of which occurred in a time course-dependent manner.

2 Materials and methods

2.1 Experimental design

Eight healthy, sedentary, males volunteered to participate in the study (23 ± 2 years, 1.79 ± 0.03 m, 75.3 ± 3.0 kg, 23.6 ± 0.9 kg m^{-2}, 13.3 ± 2.2% body fat). All experimental procedures were approved by the Dublin City University Research Ethics Committee in accordance with the Declaration of Helsinki. Each participant underwent a thorough medical screening and provided written informed consent prior to participation. Subjects were physically inactive for at least 6 months and peak oxygen uptake (VO_{2peak}, 2.81 ± 0.15 L/min) was determined by indirect calorimetry (Vmax 29C, SensorMedics, Yorba Linda, CA) using an incremental protocol on an electronically braked stationary cycle ergometer (Ergoline 900, SensorMedics). The experimental design consisted of participants cycling for 60 min per session at the target exercise intensity of 80% VO_{2peak} on 14 consecutive days. Each training session was supervised and performed in the Human Performance Laboratory at Dublin City University. VO_2 was monitored during each session as the measure of exercise intensity and the power output was adjusted accordingly to elicit the target exercise intensity. Muscle biopsies were taken in the morning prior to the first training session, and the mornings following the 7th and 14th training session. A test for peak oxygen uptake was performed 48–72 h after the last training session to measure training-induced changes in VO_{2peak}.

2.2 Skeletal muscle biopsies

Skeletal muscle specimens (approximately 200 mg of tissue) were taken by muscle biopsy from the m. vastus lateralis under local anaesthesia using the percutaneous muscle biopsy technique with suction applied [20]. A fresh incision was made for each biopsy, at least 2 cm from a previous biopsy site. Muscle samples were snap frozen in liquid nitrogen and stored at -80°C until analysis. On the morning preceding, the first training session, participants reported to the Metabolic Physiology Research Unit after an overnight (>8 h) fast, and a resting muscle biopsy was taken (baseline, day 0). On the morning after the 7th (day 7) and 14th (day 14) training sessions, participants reported again after an overnight fast for the 2nd and 3rd biopsies, respectively. These biopsies were taken 16 h after the cessation of the previous exercise training session.

2.3 Citrate synthase activity assay

Citrate synthase activity was determined in whole-muscle homogenates using a commercially available assay kit (CS0720; Sigma-Aldrich, Poole, UK), with absorbance read

at 412 nM (SpectraMax; Molecular Devices, Sunnyvale, CA, USA). Maximal activity of citrate synthase was measured as a marker of adaptation to training, i.e. altered aerobic metabolism, in skeletal muscle as classically described [6].

2.4 Preparation of a mitochondria-enriched protein fraction from skeletal muscle

All chemicals and reagents described were purchased from Sigma-Aldrich unless otherwise stated. Mitochondrial enrichment from skeletal muscle biopsies was performed using a commercially available mitochondrial isolation kit as per the manufacturer's instructions (MITO-ISO1; Sigma-Aldrich). Briefly, 50 mg of skeletal muscle was cut into small pieces and incubated on ice for 3 min in ten volumes of extraction buffer B (20 mM MOPS, pH 7.5, containing 110 mM KCl and 1 mM EGTA) containing 0.25 mg/mL trypsin. The solution was briefly centrifuged at 4°C, and the supernatant was removed by aspiration. All subsequent centrifugation steps took place at 4°C. The pellet was re-suspended in eight volumes of extraction buffer containing 0.25 mg/mL trypsin, and incubated on ice for 20 min. An albumin solution (50 mg/mL) was added to obtain a final concentration of 10 mg/mL to quench the proteolytic reaction. This solution was vortexed and briefly centrifuged. The supernatant was removed by aspiration and the pellet was washed in eight volumes of extraction buffer (without trypsin) followed by brief centrifugation. The supernatant was again aspirated and another eight volumes of extraction buffer (without trypsin) were added to the pellet. This solution was then homogenized in a dounce homogenizer (~200 rpm). Large cellular debris and nuclei were pelleted by centrifuging at $600 \times g$ for 5 min. Mitochondria were pelleted by centrifuging the supernatant for 10 min at $11\,000 \times g$. The pellet was re-suspended in 500 μL of extraction buffer, and the centrifugation steps at 600 and $11\,000 \times g$ were repeated. The resulting mitochondria-enriched protein fraction was then re-suspended in 1 × storage buffer (10 mM HEPES, pH 7.4, containing 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4, and 1 mM DTT), and stored at −80°C until analysis. Experiments and results verifying the purity and mitochondrial protein enrichment of this protein fraction are described in Supporting Information.

2.5 Protein precipitation and quantification

Mitochondrial proteins suspended in the 1 × storage buffer were precipitated using the ReadyPrep 2-D Cleanup kit (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions. The resulting pellet was re-suspended in DIGE lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris-HCl, pH 8.5). Total protein concentration was determined using the Quick-Start Bradford dye reagent (Bio-Rad).

2.6 Protein labelling for 2-D DIGE

For DIGE experiments, protein fractions from days 0, 7, and 14 were labelled with 200 pmol of either Cy3 or Cy5 fluorescent dyes (GE Healthcare, Arlington Heights, IL, USA) for comparison on the same gel. Labelling reactions were performed on ice in the dark for 30 min, and then quenched with a 50-fold molar excess of free lysine to dye for 10 min on ice. An internal standard containing a pool of all samples (days 0, 7, and 14) was labelled with Cy2 fluorescent dye, and this was used as a standard on all gels to aid image matching and cross-gel statistical analysis [14]. The Cy3- and Cy5-labelling reactions (50 μg of each) from each lysate were mixed and run on the same gels with an equal amount (i.e. 50 μg/gel) of Cy2-labelled standard. As part of the 2-D DIGE experimental design, reverse labelling (reciprocal labelling) was included to normalize the label differences.

2.7 Protein separation, image acquisition, and data analysis

Labelled mitochondrial enriched proteins were separated by 2-DE as described previously [21] (Supporting Information). All gels were scanned using the Typhoon 9400 Variable Mode Imager (GE Healthcare) to generate gel images at the appropriate excitation and emission wavelengths from the Cy2-, Cy3-, and Cy5-labelled samples. The resultant gel images were cropped using the ImageQuant software tool and imported into Decyder 6.5 software. The biological variation analysis (BVA) module of Decyder 6.5 was used to compare the control with the test samples to generate the lists of differentially expressed proteins. For statistical purposes, an ANOVA score of $p < 0.05$ was considered significant.

2.8 Protein digestion and spot selection

Preparative gels containing 400 μg of protein were fixed and then post-stained with colloidal Coomassie Blue (CCB). The CCB-stained gels were scanned using the Typhoon 9400 Variable Mode Imager using the red laser without an emission filter. The subsequent gel image was cropped (ImageQuant), imported into the biological variation analysis module of DeCyder software, and was matched to the images generated from DIGE analysis. Spots of interest were selected and a pick list was generated and imported into the software of the Ettan Spot Picker robot (GE Healthcare). Gel plugs were subsequently digested with trypsin according to the standard protocols [22] (Supporting Information).

2.9 Protein identification

Tryptic-digested proteins were analysed on a nano-LC system (Ultimate 3000; Dionex, Sunnyvale, CA, USA) coupled to an Orbitrap mass spectrometer (Thermo Fisher Scientific) in high-throughput configuration. Samples were concentrated and desalted on RPC trap columns (Zorbax 300SB C18, 0.3 mm × 5 mm; Agilent Technologies, Palo Alto, CA, USA), and the peptides were separated on a nano-RPC column (Zorbax 300SB C18, 0.075 mm × 100 mm; Agilent Technologies) using a linear ACN gradient from 0 to 65% over 60 min. Additional procedural details are described in the Supporting Information. Protein identification searches were performed using the information in the tandem mass spectra by searching against the UniProtKB/Swiss-Prot (Release 57.5) human protein database using MASCOT. Searches were done with trypsin specificity (two missed cleavages allowed), carboxyamidomethylation as fixed modification, and oxidized methionine as variable modification. Precursor ion and fragment ion mass tolerances were 10 ppm and 0.5 Da, respectively.

2.10 Validation of 2-D DIGE results by immunoblotting

To confirm LC-MS/MS identification of differentially expressed proteins, traditional 1-D SDS-PAGE followed by immunoblot analysis with specific primary antibodies towards several representative proteins was performed. Approximately 20 mg of crude muscle was freeze-dried, dissected free of blood and connective tissue, and homogenized in 50 μL/mg freeze-dried muscle of ice-cold homogenization buffer (20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, 10% w/v glycerol, 10 mM NaF, 1 mM EDTA, 5 mM sodium pyrophosphate, 0.5 mM Na_3VO_4, 1 μg/mL leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride, 1 μg/mL aprotinin, 1 mM DTT, 1 mM benzamidine, and 1 μM microcystin) using a motorized pestle. Homogenates were rotated end-over-end for 60 min at 4°C, centrifuged (12 000 × *g* for 15 min at 4°C), and the protein content of the supernatant was determined by a commercially available detergent-compatible colorimetric assay (Bio-Rad). For whole-muscle lysates, an aliquot of muscle homogenate (15 μg) was mixed with Laemmli buffer (20% glycerol, 62.5 mM Tris-HCl, 2% SDS, 0.00125% bromophenol blue, 2% β-mercaptoethanol), whereas for mitochondrial extracts, an aliquot (15 μg) of mitochondria-enriched protein fraction was mixed with 1 × Laemmli sample buffer (161-0737; Bio-Rad) with 5% β-mercaptoethanol v/v. Laemmli lysates were then separated in 1-D with SDS-PAGE on Criterion XT pre-cast 4–12% gradient Bis-Tris gels (345-0124; Bio-Rad), and transferred to PVDF membranes using the Criterion Cell and Blotter systems (Bio-Rad) as per the manufacturer's instructions (described in Supporting Information). Consistency of protein loading per lane was confirmed by Ponceau staining. Following transfer, non-specific protein binding was blocked in a 7.5% milk/2.5% BSA/TBS-t (10 mM Tris, pH 7.5, 100 mM NaCl, 0.4% Tween-20) for 2 h at room temperature. Membranes were incubated overnight with primary antibodies directed towards NADH dehydrogenase 1 α sub-complex subunit 5 (NDUA5; Proteintech, 16640-1-AP), ATP synthase β (Abcam, ab14730), malate dehydrogenase (MDH; Aviva, ARP4826), and manganese-superoxide dismutase (Mn-SOD; Abcam, ab16956) diluted in primary antibody dilution buffer (1 × TBS, 0.1% BSA, 0.1% sodium azide). Membranes were washed in TBS-t, incubated with appropriate secondary HRP-conjugated antibodies (1:20 000; Bio-Rad) diluted in 2.5% milk/TBS-t, visualized by ECL (RPN2106; GE Healthcare), and quantified by densitometry (GS800 Calibrated Imaging Densitometer; Bio-Rad).

2.11 Biological network analysis of differentially expressed proteins

Canonical pathway mapping and exploration of interaction relationships between mitochondrial targets with altered protein abundance in response to exercise training was performed using Ingenuity Pathway Analysis (IPA) (v8.7; Ingenuity Systems, Redwood City, CA, USA; www.ingenuity.com) as described in Supporting Information.

2.12 Statistical analysis

Data are presented as mean ± SEM, $n = 8$. One-way repeated measures ANOVA with post-hoc pair-wise comparisons using Fisher's least significant difference was used to investigate the effect of exercise training on mitochondrial protein abundance and citrate synthase activity across the three time points. Student's paired *t*-test was used to investigate the differences between pre- and post-training VO_{2peak} values. A significance level of $\alpha = 0.05$ was applied for all statistical tests.

3 Results and discussion

3.1 Exercise training improves aerobic capacity and remodels the skeletal muscle mitochondrial proteome

We investigated the effect of short-term endurance exercise training on global remodelling of the mitochondrial proteome from human skeletal muscle using a 2-D DIGE approach. Average exercise intensity of the 14 training sessions was 80.5 ± 1.9% of the pre-training VO_{2peak}, and resulted in 17.8 ± 3.5% increase in VO_{2peak} compared with pre-training values ($p = 0.002$, Fig. 1A). This coincided with 35% ($p = 0.010$) and 32% ($p = 0.035$) increases at day 7 and

day 14, respectively, in the maximal activity of citrate synthase (Fig. 1B), a classic marker of skeletal muscle mitochondrial adaptation to exercise training [6]. 2-D DIGE analysis revealed adaptive remodelling in response to short-term training (Fig. 2) that suggests (i) increased capacity for ATP provision and re-synthesis, (ii) increased antioxidant buffering and delivery of oxygen to the mitochondrion, and (iii) increased abundance of proteins from the molecular machinery regulating mitochondrial protein synthesis and stabilization. The mitochondrial remodelling induced by exercise training demonstrated time course-dependent induction such that many proteins were elevated after just 7 days of exercise training, whereas other proteins increased in abundance only after 14 days of training. The overall effects of these adaptive changes form the molecular basis that underpins the classically observed adaptations to training including an increase in aerobic capacity, ATP provision from oxidative metabolism and a given level of mitochondrial respiration accomplished with less perturbation of adenine nucleotides [6].

3.2 Protein expression profiling using 2-D DIGE

3.2.1 Analysis of gel images

To identify the proteins that are expressed differentially between 0, 7, and 14 days training samples, we used 2-D DIGE in concert with DeCyder differential analysis software and subsequent MS to identify the proteins of interest. A total of 800 protein spots were detected in the present study. Thirty-one gel spots were detected as significantly different from day 0 (ANOVA, $p<0.05$) in mitochondria-enriched fractions from day 7 and day 14 skeletal muscle biopsies (Fig. 3 and Tables 1 and 2). All 31 spots were examined individually on each of the analytical gels to ensure that the fluorescence represented a 'true' protein spot, and was not merged with neighbouring spots. These spots represented 27 distinct proteins, as 3 proteins were present in multiple spots, namely dihydrolipoyl dehydrogenase (DLD; spots 8–10), triosephosphate isomerase (spots 28 and 29), and TRIM72 (spots 30 and 31).

3.2.2 Protein identification

Preparative gels stained with CCB G-250 were scanned and matched to the Master gel image generated from the 2-D DIGE experiment. Table 1 summarizes the MS data for the identified proteins including the number of matched peptides, % sequence coverage, and total MASCOT score. The corresponding protein to each spot number is presented in the representative gel image (Fig. 3). As a critical factor in comparative proteomics of the mitochondria is the purity and enrichment of the prepared fraction, we performed an immunoblot survey to assess non-mitochondrial organelle and contractile protein contamination, and mitochondrial protein enrichment from fraction preparations (Supporting Information). As shown in Supporting Information Fig. S1, protein markers of the contractile apparatus (MHC I and IIa, desmin), nucleus/sarcoplasm (HDAC5, FOXO1A), and glycolytic metabolism (GSK3α, GAPDH) were depleted in skeletal muscle mitochondrial fraction preparations. For mitochondrial marker proteins, as expected an enrichment of mitochondrial protein was observed for selected marker proteins (ATP synthase β, MDH, SOD, and NDUA5). This survey confirmed the purity and mitochondrial enrichment of these fractions for DIGE analysis. Further methodology and discussion are included in the accompanying Supporting Information.

To qualitatively assess the mitochondrial localization of the DIGE-identified proteins, the list of differentially expressed proteins provided in Table 1 was cross-referenced with the recently launched searchable catalogue system MitoMiner [23], which is an integrated database of proteomic data from six species based on the published

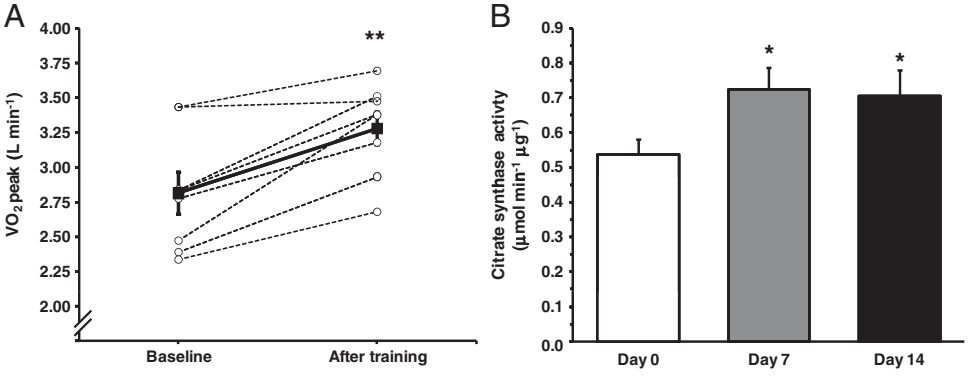

Figure 1. Increase in maximal aerobic capacity and mitochondrial enzyme activity induced by short-term endurance exercise training. (A) Individual (open circles) and group (black boxes, $n=8$) differences in maximal aerobic capacity after 14 consecutive days of endurance exercise training. (B) Maximal activity of citrate synthase determined on whole-muscle lysates at baseline (day 0) and after 7 and 14 consecutive days of endurance exercise training. $^{*}p<0.05$ versus day 0; $^{**}p<0.01$ baseline versus after training.

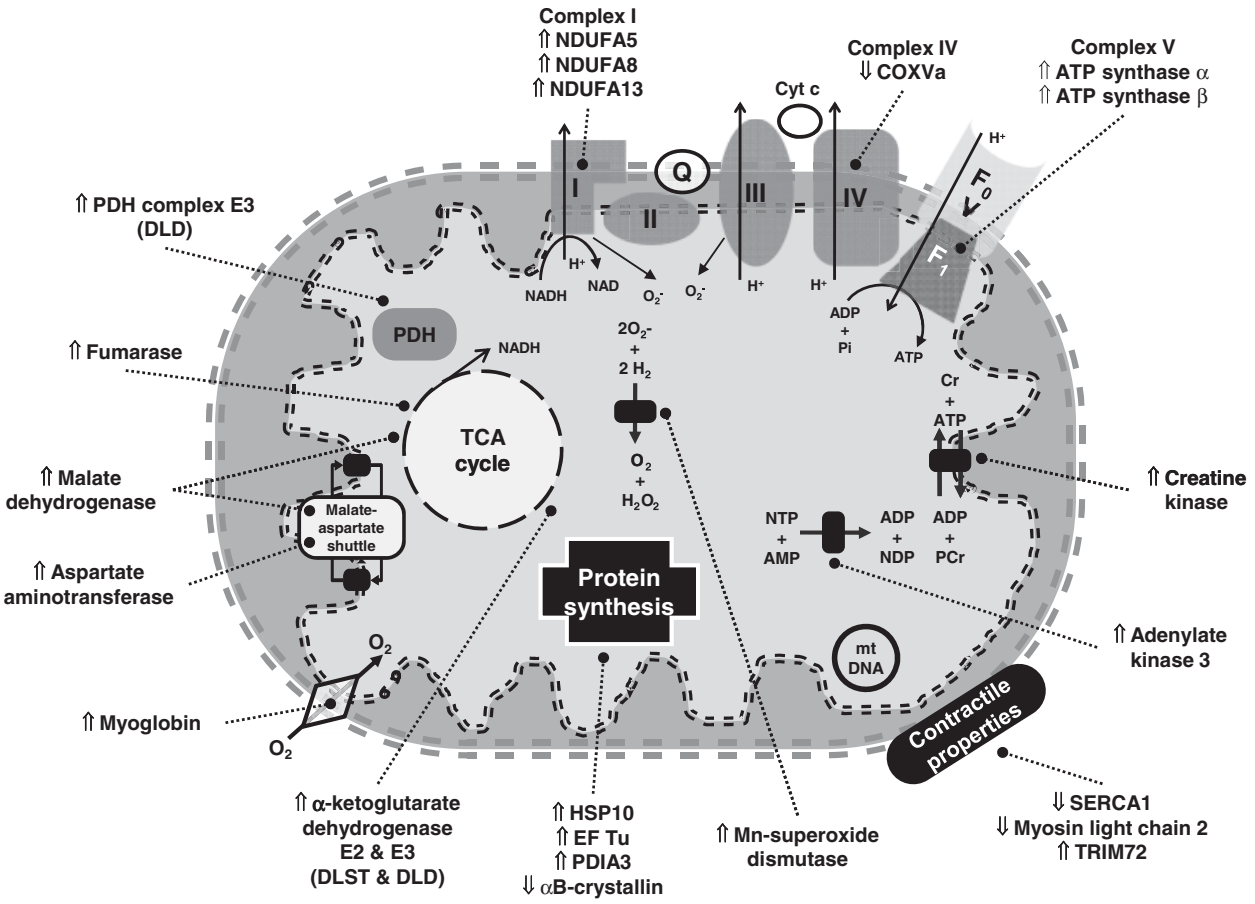

Figure 2. Schema of remodelling of mitochondrial proteome of human vastus lateralis muscle in response to short-term endurance exercise training. Proteins with increased abundance after exercise training are denoted by ⇑, whereas proteins with decreased abundance after exercise training are denoted by ⇓. Quantitative values are listed in Table 2. The exercise training-induced remodelling of the mitochondrial proteome is indicative of increased capacity for ATP provision and resynthesis, oxygen delivery and antioxidant capacity, and alterations in the molecular machinery-regulating mitochondrial protein synthesis. See text for abbreviations.

experimental data. All but three proteins, namely myosin regulatory light chain 2 (spot 19), galectin-1 (spot 15), and TRIM72 are annotated as localized to the mitochondrion in this database. The identification of proteins not classically considered localized to the mitochondrion may be interpreted as contaminating artefact of the isolation procedure although a certain amount of contamination may be acceptable in such experiments [17, 24]. On the other hand, there is an increasing acceptance that skeletal muscle mitochondria represent a difficult organelle to purify for comparative proteomics by virtue of their organization as a continuous reticulum and their association with myofilaments [25–27]. For example, proteins such as SERCA1 and aldolase, which are annotated as 'non-mitochondrial' by Gene Ontology analysis, have been identified in purified functional mitochondrial preparations by independent experiments [23, 25, 26]. In general, the presence of annotated non-mitochondrial proteins in mitochondria-enriched fractions may be tissue-specific contamination caused by the tethering of mitochondria to the myofilament apparatus, or alternatively, the localization of cytosolic proteins to the outer mitochondrial membrane.

3.2.3 Verification of 2-D DIGE results by immunoblot analysis

To verify the results of the 2-D DIGE analysis, we performed Western blot analysis on lysates from crude muscle fractions and mitochondria-enriched fractions using antibodies against NDUA5, ATP synthase β, MDH, and Mn-SOD. Representative immunoblots are included, in conjunction with graph views and 3-D simulation of the protein spots from the DeCyder analysis (Fig. 4). Immunoreactive bands for each protein were clearly observed in all mitochondria-enriched fractions from the skeletal muscle biopsies and confirmed the differential expression pattern identified by the 2-D DIGE analysis. Similar differential expression patterns were observed in whole-muscle protein lysates (Fig. 4), thereby confirming that the results obtained by

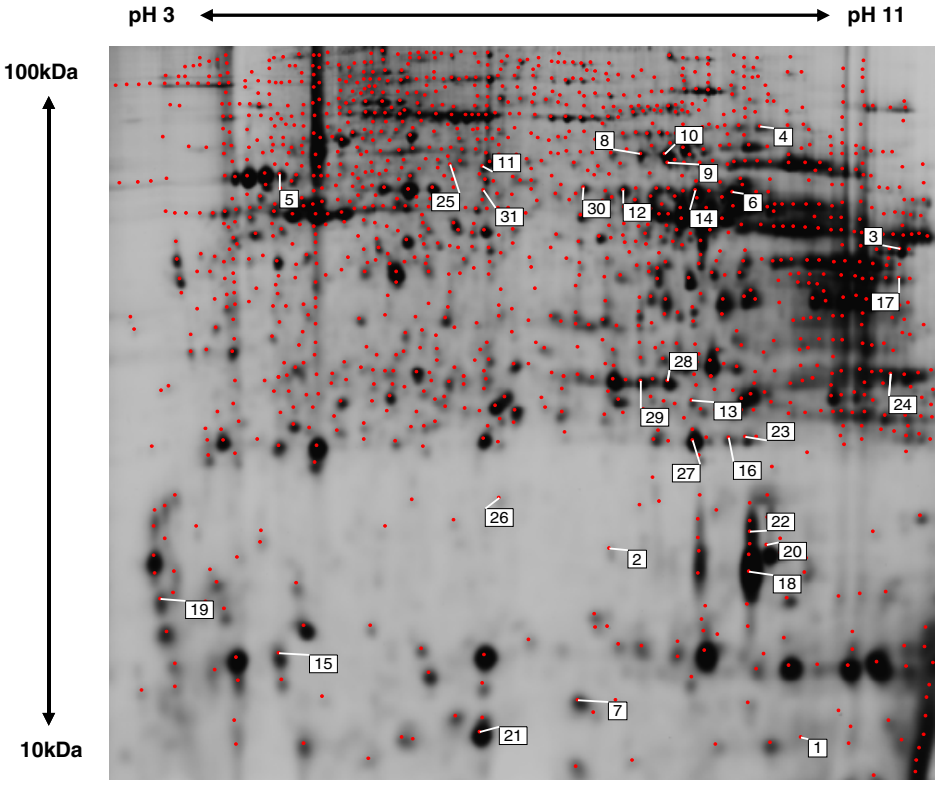

Figure 3. Differentially expressed protein spots identified by DIGE analysis. Thirty-one protein spot features (1–31) were found to be statistically different (ANOVA < 0.05) in mitochondria-enriched protein fractions from skeletal muscle biopsies after days 0, 7, and 14 of endurance exercise training. Shown is the Cy2-labelled master gel of the mitochondria-enriched protein fraction from human skeletal muscle over the pH range of 3–11. A detailed listing of proteins with altered abundance is provided in Table 1. The pH values of the first-dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated on the top and on the left of the panels, respectively.

DIGE analysis on mitochondria-enriched fractions in this experiment can be extrapolated to the whole-muscle proteome and considered in physiological context as in the subsequent sections.

3.3 Time course-dependent exercise training-induced changes in mitochondrial proteins

The time course of exercise training-induced changes in mitochondrial proteins in human skeletal muscle is poorly described. Despite an observed increase in mitochondrial content of approximately 50–100% after 6 wk of training [28], measurable changes in skeletal muscle metabolism such as enzymatic activity and substrate metabolism during exercise are observed between 3 and 14 days of training [29]. Some [18, 19], but not all [30], have demonstrated increased citrate synthase activity and mitochondrial ATP production rate, coincident with an increase in maximal oxygen consumption between 5 and 10 days of training. Although the majority of proteins altered by exercise training in the present study were elevated after both 7 and 14 days of training, alterations in protein abundance for several proteins including aldolase (spot 13), GTP:AMP phosphotransferase mitochondrial/adenylate kinase 3 (AK3; spot 16), elongation factor Tu (EF-Tu; spot 12), Mn-SOD (spot 27), myoglobin (spot 18), and sarcoendoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1, spot 26) only reached significance after 14 days of training. These patterns are likely to reflect the differences in the half-time of protein induction and the differential regulation of gene-specific transcriptional and translational processes regulated by repeated bouts of exercise. Time course-dependent alterations in mitochondrial proteins after the onset of contractile activity typically show either accelerated rates of induction initially, or a distinct lag period followed by induction on an asymptotic time course [31].

3.4 Exercise training-induced changes in electron transport chain proteins

In the present study, short-term exercise training resulted in alterations in subunit expression of complexes I (NADH: ubiquinone oxidoreductase/NADH dehydrogenase), IV (cytochrome *c* oxidase (COX)), and V (F_0F_1-ATP synthase). These findings logically suggest that exercise training enhances the capacity for electron flux through the electron transport chain (ETC) (and therefore, a larger proton gradient to support ATP synthesis by complex V), coupled to a higher capacity for ADP phosphorylation owing to increased ATP synthase α and β subunit abundance. This is consistent with the finding that short-term exercise training increases the mitochondrial ATP production rate in human skeletal muscle [19].

Complex I is the primary entry point for electrons into the ETC. These electrons are sourced from NADH molecules generated by catabolic reactions within the

Table 1. List of protein identifications by MS of tryptic in-gel digests of DIGE-identified differentially expressed spots after exercise training in human skeletal muscle

Spot no.	Entrez-Gene symbol	Gene ID	UniProtKB/Swiss-Prot ID	UniProtKB entry	Protein name	Theoretical molecular weight (kDa)	Theoretical isoelectric point (p*I*)	No. of identified peptides	% Sequence coverage	Protein probability	MASCOT score
1	HSPE1	3336	P61604	CH10_HUMAN	10 kDa HSP; mitochondrial	10 800	8.91	2	25.5	7.80E−10	281
2	CRYAB	1410	P02511	CRYAB_HUMAN	α-Crystallin B chain	20 158	6.76	9	49.1	2.20E−16	609
3	GOT2	2806	P00505	AATM_HUMAN	Aspartate aminotransferase; mitochondrial	44 695	8.98	6	15.3	1.30E−08	314
4	ATP5A1	498	P25705	ATPA_HUMAN	ATP synthase subunit α; mitochondrial	55 209	8.28	24	41.4	6.10E−13	1190
5	ATP5B	506	P06576	ATPB_HUMAN	ATP synthase subunit β; mitochondrial	51 769	5.00	28	37.4	4.00E−10	1234
6	CKMT2	1160	P17540	KCRS_HUMAN	Creatine kinase; sarcomeric mitochondrial	43 343	7.28	8	12.2	9.40E−11	242
7	COX5A	9377	P20674	COX5A_HUMAN	COX subunit 5A; mitochondrial	16 761	6.30	10	37.3	1.00E−30	849
8	DLD	1738	P09622	DLDH_HUMAN	Dihydrolipoyl dehydrogenase; mitochondrial	50 174	6.50	11	27.2	2.70E−13	391
9					Dihydrolipoyl dehydrogenase; mitochondrial			26	44.2	1.30E−14	1255
10					Dihydrolipoyl dehydrogenase; mitochondrial			13	23.0	1.00E−30	864
11	DLST	1743	P36957	ODO2_HUMAN	Dihydrolipoyllysine-residue succinyltransferase	41 363	5.90	10	20.2	1.00E−30	539
12	TUFM	7284	P49411	EFTU_HUMAN	Elongation factor Tu; mitochondrial	45 045	6.31	14	37.6	1.00E−11	685
13	ALDOA	226	P04075	ALDOA_HUMAN	Fructose-biphosphate aldolase A	39 288	8.39	5	19.0	1.10E−07	226
14	FH	2271	P07954	FUMH_HUMAN	Fumarate hydratase; mitochondrial	50 081	6.99	4	8.6	2.50E−07	241
15	LGALS1	3956	P09382	LEG1_HUMAN	Galectin-1	14 584	5.34	7	67.4	1.60E−09	438
16	AK3	205	Q9UIJ7	KAD3_HUMAN	GTP:AMP phosphotransferase mitochondrial	25 434	9.16	7	31.3	1.22E−09	311
17	MDH2	4191	P40926	MDHM_HUMAN	MDH; mitochondrial	33 000	8.54	6	29.6	1.50E−11	582
18	MB	4151	P02144	MYG_HUMAN	Myoglobin	17 052	7.29	12	64.9	4.00E−12	821
19	MYLPF	29895	Q96A32	MLRS_HUMAN	Myosin regulatory light chain 2, skeletal muscle isoform	18 883	4.91	17	56.2	2.00E−11	511
20	NDUFA13	51079	Q9P0J0	NDUAD_HUMAN	NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 13	16 567	8.24	2	13.2	3.00E−07	398
21	NDUFA5	4698	Q16718	NDUA5_HUMAN	NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 5	13 328	5.77	8	55.2	2.00E−14	475
22	NDUFA8	4702	P51970	NDUA8_HUMAN	NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 8	19 973	7.93	3	24.4	2.70E−09	474
23	PEBP1	5037	P30086	PEBP1_HUMAN	Phosphatidylethanolamine binding protein	20 925	7.43	11	75.4	6.10E−13	1053
24	PGAM2	5224	P15259	PGAM2_HUMAN	Phosphoglycerate mutase 2	28 634	9.00	12	38.7	6.70E−15	440

Table 1. Continued

Spot no.	Entrez-Gene symbol	Gene ID	UniProtKB/Swiss-Prot ID	UniProtKB entry	Protein name	Theoretical molecular weight (kDa)	Theoretical isoelectric point (p*I*)	No. of identified peptides	% Sequence coverage	Protein probability	MASCOT score
25	PDIA3	2923	P30101	PDIA3_HUMAN	Protein disulfide isomerase A3	54 265	5.61	14	38.2	5.00E−11	576
26	ATP2A1	487	O14983	AT2A1_HUMAN	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	110 252	5.07	7	6.5	1.30E−09	406
27	SOD2	6648	P04179	SODM_HUMAN	Superoxide dismutase (Mn); mitochondrial	22 204	6.86	4	30.2	1.80E−13	227
28	TPI1	7167	P60174	TPIS_HUMAN	Triosephosphate isomerase	26 538	6.51	16	75.9	1.00E−14	1677
29					Triosephosphate isomerase			13	71.5	8.90E−15	1467
30	TRIM72	493829	Q6ZMU5	TRI72_HUMAN	Tripartite motif-containing protein 72	52 730	6.05	14	35.0	1.50E−12	513
31					Tripartite motif-containing protein 72			20	35.6	5.10E−13	640

Spot reference numbers are consistent throughout the article. The table shows protein name/identification, corresponding gene name and ID, theoretical molecular mass, theoretical p*I*, number of peptides sequenced, percent sequence coverage, protein probability, and MASCOT scores for each of the 31 differentially expressed protein spots in mitochondria-enriched protein fractions from untrained compared to trained human skeletal muscle. Theoretical molecular weight and p*I* values are calculated from the UniProtKB/Swiss-Prot database.

mitochondrial matrix. Three subunits of complex I [NDUA5 (spot 21), NDUA8 (spot 22), and NDUA13 (spot 20)] showed the largest magnitude of increased protein abundance of all differentially regulated proteins identified (Table 2). This supports the observation that complex I is the primary ETC complex undergoing adaptive remodelling with high-intensity exercise training [32].

We also observed a reduction in abundance of the complex IV subunit COXVa (spot 7) in the terminal complex of the respiratory chain. The functional consequences of the reduction in COXVa protein is unknown at present. An increase in COX complex activity has classically been observed in response to exercise training [7]. Recently, 2 wk of high-intensity interval training was reported to increase maximal COX activity with concomitant increases in COXII and COXIV subunit expression [33]. Indeed, we have also observed a 30% increase in COXIV protein expression in whole-muscle lysates from the present study (Egan et al., unpublished observations). We speculate that depending on a given physiological stimulus, it is likely that the changes in protein abundance occur preferentially in few subunits rather than unidirectionally in the whole complex. Whether the reduction in COXVa activity in the present study represents the likelihood of increased or decreased COX activity is unknown. COXVa expression is necessary for COX function in yeast [34], but reducing subunit VIb expression increases mammalian complex IV activity [35]. Thus, the relative protein change in an individual subunit may not be sufficient to infer changes in whole complex function.

The major site of ATP synthesis in a eukaryotic cell occurs via oxidative phosphorylation, whereby complex V phosphorylates ADP to form ATP, a reaction catalysed by ATP synthase, is essentially powered by the proton gradient formed by electron flux through ETC complexes I–IV. We observed an approximately 50% increase in protein abundance of both α and β subunits of ATP synthase (spots 4 and 5, respectively), suggesting an enhanced capacity for ATP synthesis through oxidative phosphorylation in response to exercise training. These subunits were recently shown to be elevated (32 and 55%, α and β, respectively) in a whole-muscle proteomic analysis in response to 6 wk of treadmill interval training [15]. Of clinical relevance, protein abundance of ATP synthase β is reduced in skeletal muscle of T2DM patients [9, 12] and with aging [10]. Similarly, a general reduction in protein abundance of complexes I, III, and IV is present in insulin-resistant skeletal muscle [9].

3.5 Exercise training-induced changes in tricarboxylic acid cycle proteins and metabolic enzymes

We observed an increase in protein abundance for several enzymes involved in the tricarboxylic acid (TCA) cycle, the E3 subunit of the pyruvate dehydrogenase (PDH) complex,

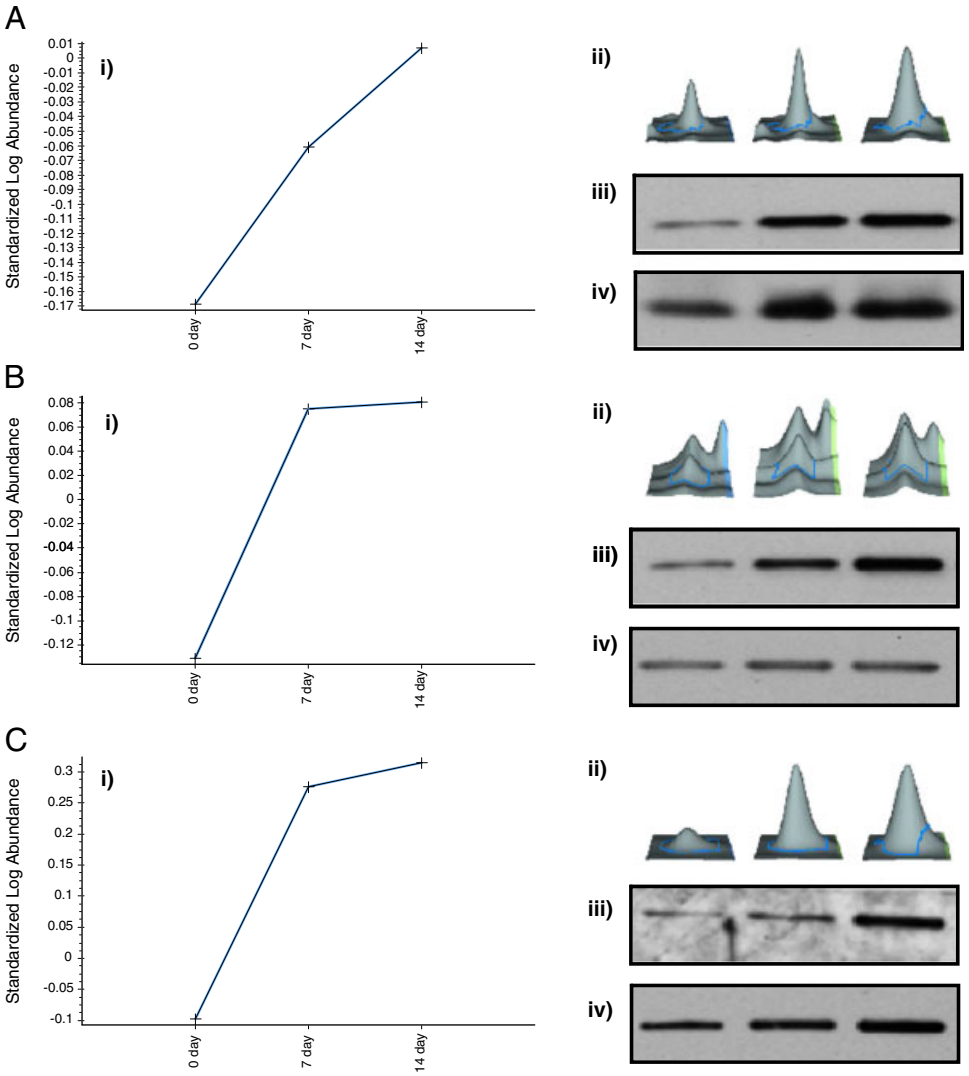

Figure 4. Representative data showing increased abundance of selected mitochondrial proteins after endurance exercise training. 2-D DIGE and Western blot analysis of (A) NADH dehydrogenase (ubiqunone) 1 α subcomplex subunit 5 (spot 21), (B) superoxide dismutase (spot 27), and (C) MDH (spot 17) in mitochondria-enriched protein fractions from skeletal muscle biopsies after days 0, 7, and 14 of endurance exercise training. The volume of each protein spot was calculated using DeCyder software and is graphically represented. Each panel includes: (i) graph view of DeCyder analysis, (ii) 3-D simulation view of DeCyder analysis, and representative immunoblot from Western blot analysis of corresponding protein from (iii) mitochondria-enriched protein fractions, and (iv) whole-muscle protein lysates.

as well as mitochondria-associated glycolytic enzymes. This pattern suggests that exercise training enhances the capacity for substrate utilization through improved use of pyruvate and increased formation of TCA cycle intermediaries that would consequently have the overall effect of providing a sufficient amount of reduced cofactors (NADH and $FADH_2$) to the respiratory chain, and ultimately contribute to enhanced oxidative phosphorylation under exercising conditions.

The TCA cycle enzymes fumarate hydratase (fumarase; spot 14) and MDH (spot 17) catalyse the sequential hydration of fumarate to malate, and oxidation of malate to oxaloacetate, with the latter oxidation reaction generating one of the three NADH molecules produced by each cycle and used in the ETC. Dihydrolipoyllysine-residue succinyltransferase (DLST; spot 11) and DLD constitute the E2 and E3 components, respectively, of the α-ketoglutarate dehydrogenase complex, a TCA cycle complex that catalyses the oxidative carboxylation of α-ketoglutarate to succinyl CoA. In addition, DLD functions as the E3 component of the PDH complex that, similar to the α-ketoglutarate dehydrogenase complex, generates NADH during the processes that ultimately produce acetyl CoA. The plasticity of MDH in skeletal muscle has been previously established as it's enzymatic activity in skeletal muscle is elevated in athletes compared with controls [36], and increased by exercise training [37]. Fumarase has also been used a marker of muscle oxidative capacity adaptation to training [38], but to our knowledge this is the first study to demonstrate an increase in abundance of both of these TCA cycle enzymes in response to short-term exercise training in human skeletal muscle.

MDH is a principal component of the malate–aspartate shuttle of the mitochondrial inter-membrane space, which transports reducing equivalents formed by glycolysis across the inner mitochondrial membrane where they contribute to the electron flux of the ETC. We also observed an exercise training-induced increase in protein expression of another

Table 2. Exercise training-induced changes protein abundance in mitochondria-enriched protein fractions from human vastus lateralis skeletal muscle

Spot no.	Protein name	One-way ANOVA *p*-Value	Fold change Day 7/day 0	Pairwise comparison *p*-Value	Fold change Day 14/day 0	Pairwise comparison *p*-Value	Av. ratio Day 14/day 7	Pairwise comparison *p*-Value
	OXPHOS and ETC							
21	NADH dehydrogenase (ubiquinone) 1 α sub-complex subunit 5	0.0004	2.32	0.0008	2.64	0.0011	1.14	0.6800
22	NADH dehydrogenase (ubiquinone) 1 α sub-complex subunit 8	0.0006	2.05	0.0005	2.12	0.0033	1.04	0.9300
20	NADH dehydrogenase (ubiquinone) 1 α sub-complex subunit 13	0.0016	1.49	0.0086	1.55	0.0019	1.04	0.6900
7	COX sub-unit 5A; mitochondrial	0.0290	−1.44	0.0200	−1.49	0.0190	−1.03	0.7400
4	ATP synthase sub-unit α; mitochondrial	0.0400	1.46	0.0480	1.52	0.0420	1.04	0.9000
5	ATP synthase sub-unit β; mitochondrial	0.0290	1.38	0.0370	1.55	0.0260	1.12	0.5700
	Substrate utilisation and TCA cycle							
28	Triosephosphate isomerase	0.0350	1.30	0.0210	1.27	0.0480	−1.09	0.1100
24	Phosphoglycerate mutase 2	0.0210	1.23	0.0130	1.17	0.0700	−1.05	0.3500
13	Fructose-biphosphate aldolase A	0.0280	1.34	0.0870	1.57	0.0250	1.17	0.2900
3	Aspartate aminotransferase; mitochondrial	0.0280	1.26	0.0200	1.30	0.0270	1.04	0.8100
11	Dihydrolipoyllysine-residue succinyltransferase	0.0140	1.59	0.0120	1.45	0.0390	−1.09	0.5000
8	Dihydrolipoyl dehydrogenase; mitochondrial	0.0055	1.45	0.0030	1.32	0.0340	−1.10	0.2700
14	Fumarate hydratase; mitochondrial	0.0210	1.43	0.0320	1.35	0.0440	−1.06	0.5700
17	MDH; mitochondrial	0.0370	1.52	0.0450	1.58	0.0360	1.04	0.9400
	Phosphotransfer system							
6	Creatine kinase; sarcomeric mitochondrial	0.0250	1.50	0.0410	1.35	0.0380	−1.11	0.4900
16	GTP:AMP phosphotransferase mitochondrial	0.0020	1.20	0.2300	1.68	0.0018	1.40	0.0073
	Oxygen transport							
18	Myoglobin	0.0350	1.07	0.6100	1.43	0.0260	1.35	0.0470
	Oxidative stress response							
27	Superoxide dismutase (Mn); mitochondrial	0.0370	1.24	0.1600	1.44	0.0210	1.16	0.1900
	Protein synthesis machinery							
1	10 kDa HSP; mitochondrial	0.0430	1.48	0.0800	1.60	0.0310	1.08	0.7000
12	Elongation factor Tu; mitochondrial	0.0360	1.14	0.1300	1.22	0.0280	1.07	0.2300
2	α-Crystallin B chain	0.0180	−1.63	0.0580	−1.81	0.0110	−1.11	0.6000
25	Protein disulfide isomerase A3	0.0450	1.37	0.0570	1.33	0.0510	−1.03	0.7600
	Other							
26	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	0.0025	−1.18	0.3200	−1.65	0.0027	−1.40	0.0039
19	Myosin regulatory light chain 2, skeletal muscle isoform	0.0002	−1.48	0.0006	−1.47	0.0006	1.01	0.9100
15	Galectin-1	0.0220	−1.38	0.0095	−1.28	0.0480	1.08	0.5300
30	Tripartite motif-containing protein 72	0.0028	1.33	0.0077	1.30	0.0038	−1.03	0.7300
23	Phosphatidylethanolamine binding protein	0.0014	1.52	0.0021	1.39	0.0068	−1.09	0.3500
	Multiple spots							
31	Tripartite motif-containing protein 72	0.0140	1.43	0.0230	1.70	0.0120	1.19	0.4000
29	Triosephosphate isomerase	0.0140	1.38	0.0280	1.18	0.0790	−1.10	0.3200
9	Dihydrolipoyl dehydrogenase; mitochondrial	0.0270	1.26	0.0350	1.29	0.0370	1.01	1.0000
10	Dihydrolipoyl dehydrogenase; mitochondrial	0.0430	1.37	0.0940	1.26	0.0480	−1.06	0.4900

Proteins are manually categorized based on known protein function from UniProtKB/Swiss-Prot and Entrez-Gene databases. Pairwise comparison represents *p*-value from Fisher's LSD post-hoc test for each comparison subsequent to the one-way ANOVA: day 7 versus day 0, day 14 versus day 0, and day 14 versus day 7. Day 7/day 0 indicates the fold change in protein abundance from day 7 of the training programme relative to day 0. Day 14/day 0 indicates the fold change in protein abundance from day 14 of the training programme relative to day 0. Day 14/day 7 indicates the average ratio between the fold change from day 14 to day 0 and day 7 to day 0.

key enzyme in this pathway of aspartate aminotransferase (mAspAT; spot 3), consistent with the previous reports [39, 40]. An increase in the capacity of the malate–aspartate shuttle and therefore greater oxidation of NADH, coupled with greater capacity through PDH complex and therefore less pyruvate reduction to lactate, has been proposed as the mechanistic basis for reduced lactate formation during submaximal exercise subsequent to a period of exercise training [6].

Three enzymes of glycolytic pathway namely triosephosphate isomerize, phosphoglycerate mutase 2 (spot 24), and aldolase A were elevated after training. Although glycolytic enzymes are traditionally considered cytoplasmic, there has been an increasing proteomic evidence that these enzymes can be localized to the outer mitochondrial membrane, and thus, are designated as 'mitochondrial' in the studies that have isolated functional mitochondria [25, 26]. Increased protein expression of glycolytic and TCA enzymes is again consistent with the increments in the capacity for bioenergetic activity in muscle as an adaptive response to exercise training.

3.6 Exercise training-induced changes in the phosphotransfer system

The increases in mitochondrial creatine kinase (mi-CK; spot 6) and AK3 suggest a reconfiguration of the system for phosphate transfer and ATP re-synthesis. The studies of mitochondria isolated from endurance-trained muscle have revealed a reduced sensitivity to ADP-stimulated respiration [41], but an enhanced creatine-stimulated response [42]. mi-CK is located in the inter-membrane space and is coupled to the adenine nucleotide translocase on the inner membrane. By catalysing the phosphorylation of creatine (Cr) to phosphocreatine (PCr) from newly generated ATP [43], mi-CK provides (i) ADP for oxidative phosphorylation and ATP transport out of the mitochondria and (ii) PCr for anaerobic ATP production in the cytosol. mi-CK has a direct role in respiratory function as CK-deficient animals (mitochondrial and cytosolic) have a greater impairment in voluntary running capacity compared with wild-type or cytosolic CK-deficient animals [44]. In addition, the Cr/PCr ratio and the availability of ADP are important regulators of mitochondrial respiration. The apparent K_m for ADP is higher in exercise-trained individuals and is decreased with the addition of creatine [45]. These results suggest that the PCr/Cr ratio, as determined by mi-CK, may be a more important regulator of mitochondrial respiration than ADP following exercise training.

AK3 is a member of the phosphotransferase family of adenylate kinases that contribute to the homeostasis of adenine nucleotides. AK3 is exclusively located in the mitochondrial matrix and phosphorylates AMP using GTP and ITP (GTP+AMP↔GDP+ADP) as phosphoryl donors [46]. The role of AK3 following exercise training has not been described, but it is proposed to regulate mitochondrial GTP produced by succinyl CoA synthethase during the TCA cycle [47]. The greater AK3 protein content following exercise training may help maintain mitochondrial function by increasing GDP as a phosphoryl acceptor for the TCA cycle and the availability of ADP for oxidative phosphorylation. The combined effects of mi-CK and AK3 during mitochondrial remodelling may regulate nucleotide homeostasis and phosphate transfer during subsequent exercise. Increasing aerobic performance requires an enhancement of both muscle oxidative capacity and mechanisms of respiratory control [45, 48], attesting to the importance of temporal co-ordination of energy fluxes and adenine nucleotides by phosphotransfer proteins.

3.7 Exercise training-induced changes in the mitochondrial protein synthesis machinery

Mitochondrial biogenesis refers to an increase in mitochondrial volume and alterations in organelle composition and is a well-established consequence of endurance training [49]. A new steady state of higher mitochondrial content is generally established after 6 wk of endurance training [28], but mitochondrial proteins, often with short half lives, can increase after 1 wk of contractile activity [31, 50], as observed in the present study. Many mitochondrial proteins are nuclear encoded, and translocated into the respective mitochondrial sub-compartments. The regulation of mitochondrial assembly through protein import is critical to the function of the mitochondrion [28]. Our analysis reveals exercise training-induced changes in the protein abundance of several regulators of mitochondrial protein synthesis, including two HSP family members (HSP10 and αB-crystallin; spots 1 and 2), the elongation factor EF-Tu and the protein disulfide isomerize A3 (spot 25).

αB-crystallin is a member of the small HSP family that plays a role in skeletal muscle remodelling through induction as a stress response to various stimuli including contractile activity [51]. However, the observed decrease in αB-crystallin protein is in contrast to other reports that show increased protein after acute contractile activity [52], and a period of endurance training [53]. The discrepancy between the results is unexplained at present, but may relate to the duration of the exercise training programme, which is of much shorter duration than others reported. Hence, there may be a biphasic response of this HSP, where an initial decrease in protein abundance is followed by a compensatory increase at a later time point during training. Conversely, another HSP, HSP10, was increased by exercise training at both 7 and 14 days. This protein is a member of the heat shock chaperonin family, which in concert with HSP60 is responsible for the refolding of mature proteins to an active state after mitochondrial import as precursor proteins [54]. Transgenic mice overexpressing HSP10 are protected against age-related skeletal muscle force loss and

atrophy through the prevention of apoptosis by preventing accumulation of oxidized mitochondrial proteins and, thus, maintaining mitochondrial function [55]. The translation elongation factor EF-Tu is a GTPase, localized to mitochondrial ribosomes [56], which delivers amino-acylated tRNAs of the mitochondrion to the ribosome during the elongation step in mitochondrial protein synthesis [57]. A secondary role for EF-Tu as a mitochondrial chaperone that prevents thermal aggregation of proteins and enhances protein refolding has been identified [56]. Our results suggest that this protein may play a role in exercise training-induced mitochondrial protein synthesis. The protein disulfide isomerase A3, also known as ERp57, is a member of the protein disulfide isomerase family of oxidoreductases essential for correct disulfide bond formation of newly synthesized glycoproteins and catalyzing the rate-limiting steps in the folding pathway of polypeptides [58]. Although traditionally associated with the endoplasmic reticulum, ERp57 is localized to the mitochondrion where it interacts with mitochondrial calpains in the regulation of apoptosis [59], but the significance of an exercise training-induced increase in ERp57 is unknown. However, taken together, the modulation of protein expression for this subset of proteins suggests that the molecular machinery regulating mitochondrial protein synthesis undergoes remodelling by exercise training that may be permissive in regulating part of the adaptive response of mitochondrial proteins to the training stimulus.

3.8 Exercise training-induced changes in antioxidant capacity and oxygen transport

The increased protein content of Mn-SOD suggests a greater antioxidant capacity in the mitochondria following exercise training. SOD catalyzes the removal of superoxide radicals produced by complex I and complex III of the ETC ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). Mn-SOD is located in the mitochondrial matrix and is responsible for the removal of anions formed by complex I and complex III on the matrix side [60]. Heterozygous or homozygous knockout of Mn-SOD results in a decrease in exercise capacity, contractile force, and ATP production in skeletal muscle [61]. Our results are in agreement with the reported increases in Mn-SOD following exercise training [62]. Further studies are required to determine the exact role of Mn-SOD in mitochondrial adaptation to training, but as cellular redox flux can regulate gene expression, alterations in antioxidant buffering capacity may also have a regulatory role in mitochondrial biogenesis [63].

Myoglobin is a hemoprotein localized to the outer mitochondrial membrane of striated muscle, whose roles include providing a reservoir of readily accessible oxygen, buffering intracellular oxygen concentration, facilitating intracellular oxygen transport, inactivating nitric oxide, and scavenging ROS [64]. An increase in skeletal muscle myoglobin content, as observed in the present study, plays a role in the adaptive improvement in capacity for aerobic metabolism that occurs with endurance training. A contractile activity-induced increase in myoglobin protein content has been observed previously in rodent models of exercise training [65] and chronic electrostimulation [66], but not consistently in human training studies [67]. The induction of myoglobin protein at a different rate than other mitochondrial marker proteins, as observed in the present study, is established in rodent muscle [65], and is likely to be an adaptive response to increased oxygen demand elicited by the enhanced oxidative capacity of the exercising muscle.

3.9 Exercise training-induced changes in the mitochondrial protein associated with myofibrillar contractile properties

The alterations in a cluster of proteins namely SERCA1, myosin regulatory light chain 2, and TRIM72 are evidence of a fundamental remodelling of the contractile elements associated with mitochondria of exercise-trained muscle. These results suggest an attenuation of contractile properties of fast-twitch fibres indicative of an enhanced oxidative fibre phenotype. The likely effect of these changes is to enhance the efficiency of contractile processes at submaximal exercise intensities, thereby protecting cellular homeostasis during prolonged exertion [68]. SERCA1 plays an essential role in contraction through its function in the regulation of the calcium homeostasis that is permissive to actin-myosin cross-bridge cycling. After myofibrillar contraction, SERCA1 performs the critical function of promoting muscle relaxation by sequestering Ca^{2+} from the sarcoplasm at the expense of ATP hydrolysis [69]. SERCA1 is the predominant isoform in type II muscle fibres, whereas SERCA2 is the predominant isoform in type I muscle fibres [69], and are known to be localized in areas of contact between the sarcoplasmic reticulum and the outer mitochondrial membrane [25, 26, 70]. We observed a decrease in SERCA1 protein abundance after 14 days of exercise training. This is consistent with finding from the longer training protocols [68] and from electrically simulated skeletal muscle [71]. Myosin regulatory light chains form part of the myosin molecule and thus, are key proteins in the contractile apparatus of skeletal muscle by virtue of their regulation of the speed of shortening contractions [72]. Little is known about their expression in response to training, but we observed a decrease in the protein abundance of 'fast' skeletal muscle isoform after training. Similar decreases have been observed in rabbit skeletal muscle undergoing CLFS during the transition from fast-to-slow fibre type characteristics [71]. TRIM72 is proposed as a negative-size regulator in skeletal muscle, whose expression is highest in oxidative muscle fibres [73]. The role of TRIM72 in muscle adaptation to exercise is unknown at present, but during muscle differentiation, TRIM72 expression is induced

coincident with other genes associated with oxidative metabolism including MHC I and IIa and myoglobin [73]. The promoter region of TRIM72 also contains a MEF2 binding site [73], a transcription factor that is a key regulator of the oxidative remodelling of skeletal muscle fibres [74].

4 Concluding remarks

The main effects of the exercise training intervention on mitochondrial protein abundance (Fig. 2) are consistent with an improved capacity for ATP provision and re-synthesis and reduced homeostatic perturbation in working muscle. This proteome analysis has identified several proteins that offer potential therapeutic targets for the treatment of metabolic disease. The proteins identified in this analysis that are increased in response to exercise training have also been shown to be downregulated in aged and diabetic muscle including ATP synthase α and β, ETC complexes I and IV, creatine kinase, and MDH [9, 10, 12]. In addition, enhancing the capacity of the malate–aspartate shuttle has been proposed as part of the mechanistic basis for the exercise-induced amelioration of metabolic dysfunction [40]. Further study is necessary to determine the functional role of the exercise-training responsive proteins such as TRIM72, EF-Tu, and protein disulfide isomerase A3 as regulatory proteins in mitochondrial remodelling. Our analysis of exercise training-induced mitochondrial remodelling provides a mechanistic basis for physical activity as a countermeasure for age-related declines in muscle function [10] and physical inactivity-associated metabolic disease progression [3].

This work was supported by a grant from the National Institute for Cellular Biotechnology (P.D., D.O'G.), the Irish Research Council for Science, Engineering and Technology (D.O'G.) and the European Foundation for the Study of Diabetes/Novo Nordisk European Clinical Research Programme in Adolescents with Type 2 Diabetes (D.O'G.), the Strategic Research Program in Diabetes at Karolinska Institutet (J.R.Z.), Swedish Research Council (J.R.Z.), and the European Research Council Advanced Grant Ideas Programme (J.R.Z.). The authors acknowledge the excellence in the collection of muscle biopsies provided by Dr. Noel McCaffrey, Dr. Ray Walls, and Dr. Gavin McHugh, and Professor Martin Clynes (NICB) for his role in establishing and facilitating this collaboration and for scientific input throughout this study.

The authors have declared no conflict of interest.

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